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Review article

Thermal stability of cytokines: A review

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ABSTRACT

Background: The role of cytokines in various disease states is a burgeoning field of academic study and clinical application, however there are no consensus documents on how certain cytokines should be stored prior to quantification. This information is especially of interest to researchers assembling a biobank or clinicians who have to transport specimens to a different location in order to be tested.
Objective: To review the literature and synthesize prior findings on cytokine storage and freeze/thaw stability.
Design: We searched PubMed for articles related to cytokine storage stability. All articles were analyzed for cytokines studied, source of reported cytokine concentration (i.e., human whole blood or serum, concentrations from other species or bodily sources were excluded), and reported statistical results.
Results: We identified and synthesized results of 23 peer-reviewed articles which published data on the storage and freeze/thaw stability of 33 different cytokines and chemokines.
Conclusion: There is a wide variety of reported cytokine storage and freeze/thaw stability. Interleukin-6 and tumor necrosis factor alpha are the most widely studied cytokines in regard to temperature stability. In a few cytokines, a clear consensus can be reached as to storage safety at particular temperatures, but in most, more research needs to be done and we advise the clinician or researcher to use caution in interpreting cytokine concentration results after a long period of storage or several freeze/thaw cycles.

1. Introduction

Over the past 40 years our understanding of the role of cytokines in the immune system has expanded rapidly [1], and along with that has our ability to measure cytokines in blood. A growing body of research has noted the clinical utility of accurate cytokine measurements to predict everything from clinical outcomes in sepsis [2], likelihood of schizophrenic patients to respond to medical treatment [3], development of Alzheimer's disease in the elderly [4], acceptance of transplant organs [5], to bacterial infection in children [6]. With growing utility for serum cytokine measurements both clinically and in research come questions about the relationship between storage conditions and freeze-thaw cycles and accuracy of results. This paper will review current literature referencing this area of study and summarize findings. Its purpose is to serve as a reference material for the clinician or researcher who needs accurate assays of cytokine concentrations in patient blood.

Cytokines are proteins that have autocrine or paracrine signaling mechanisms and are involved in promoting proliferation, differentiation, and regulation of hematopoietic cells and other cells with host defense functions; therefore determining the nature of an immune response (Fig. 1) [7]. Chemokines are a group of structurally related chemotactic agents for specific leukocytes that retain 4 cysteine residues which form disulfide bonds important for their tertiary structure [8]. The most studied in regards to temperature stability are a subgroup known as CC chemokines, named because the first two cysteines are in a row (as opposed to CXC chemokines, where an amino acid is between). Due to the rapid expansion in research on cytokines, it is critical to know the concentration of various cytokines circulating in a patient to gain information about a particular disease state. Currently, there are many barriers to researchers creating normative cytokine levels; amongst them the variety of cytokine quantification platforms, whether the patient's blood is collected in a tube with an anticoagulating agent, the sterility of the sample, the storage conditions of the blood prior to being analyzed, the number of freeze/thaw cycles the blood is exposed to prior to analysis, and the process of degradation of the cytokine being quantified [9] HYPERLINK "SPS:refid::bib9" .

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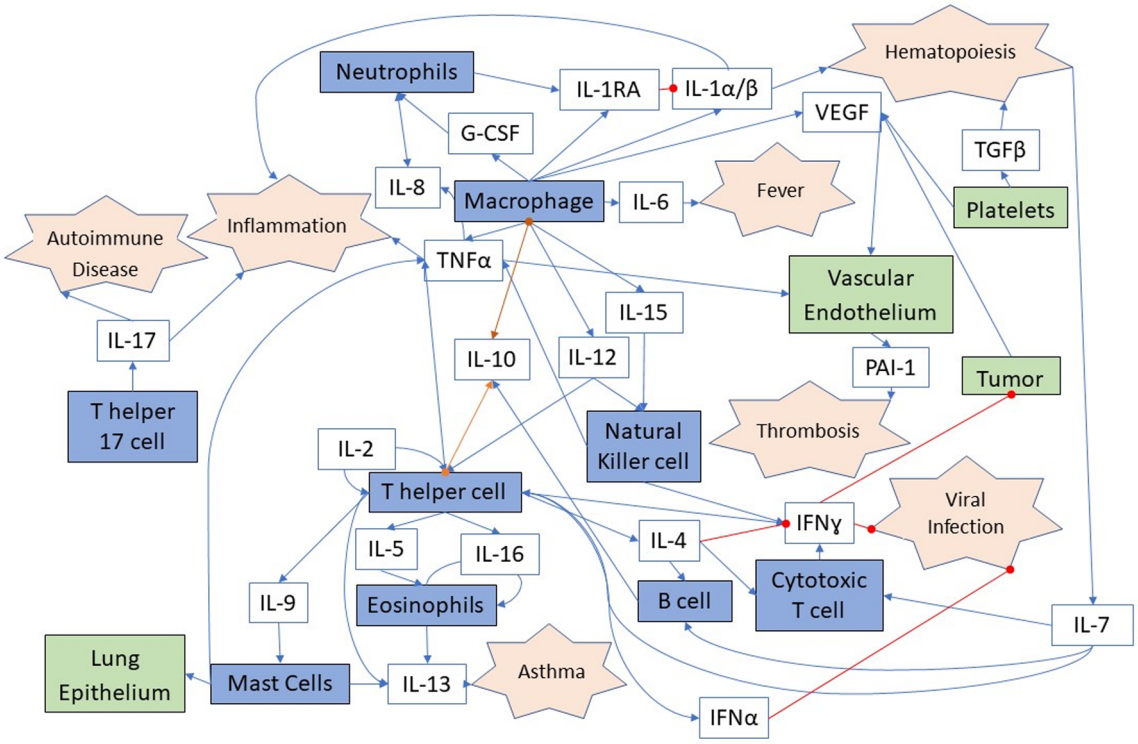

Fig. 1. Diagram showing interrelatedness of cytokines and their role in the immune system discussed in this paper. Boxes with a white background are cytokines. Boxes with a blue background are immune cell types. Boxes with a green background are non-immune cell types. Orange call-outs are clinical responses. Blue lines with arrows represent a positive correlation, orange lines with circle represent a negative correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Cytokine Assay Platforms referenced in studies and commercial manufacturers.

Type of platform	Commercial name	Commercial manufacturer	Location
Enzyme linked immunosorbent assay (ELISA)	N/A	R&D Systems	Oxon, UK
		Eurogenetics	Tessenderlo, Belgium
		Endogen	Cambridge, Mass
		Immunotech	Marseilles, France
		Genzyme	Cambridge, Mass
Immunoradiometric Assay	N/A	Medgenix	Brussels, Belgium
Bead-based multiplex immunoassay	BIO-Plex 200	Bio-Rad Laboratories	Hercules, CA
	Luminex	EMD Millipore	Billerica, MA
Chip-based multiplex immunoassay	EVIDENCE 180 Analyzer	Randox Laboratories	Crumlin, UK
	Quantibody 18-Plex	Raybiotech	Norcross, GA
Electrochemiluminescence	Meso Scale Discovery Assays	MSD	Rockville, MD
	Immulite Automated Analyzer	DPC Biermann	Bad Nauheim, Germany

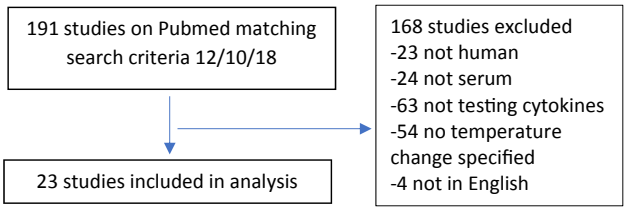

Fig. 2. Flow Diagram of PubMed Search and included studies for analysis.

The purpose of this paper is not to establish reference ranges for various cytokines in a multitude of health conditions, but rather to examine the effects of storage and transport conditions on cytokine levels, looking at each cytokine individually. Drawing on previous published data and analyses, we offer summary statements on the stability of cytokine measurements in samples collected using different blood collection tubes, stored at different temperatures for varying lengths of time, and assayed using different cytokine quantification platforms. This is necessary to assist both future researchers in the area of immunological contributions to disease, and also in physicians practicing direct patient care.

Each platform for cytokine quantification produces results that are consistent within the platform itself, but not necessarily with other assays (Table 1). Since each study utilized one type of assay for each cytokine measured, and we are comparing only changes in cytokines reported as significant within a study, we will only briefly touch on the

Table 2
Master list of studies yielded from PubMed search. Except where indicated in "comments" column, frozen means −80C, RT = room temperature, LPS = lipopolysaccharides, PHA = phytohemagglutinin.

First author and year	Population and sample size	Assay	Statistical tool used in analyses	Statistical significance	Comments
Aguilar-Mahecha [22]	14 healthy volunteers	Bead-based multiplex immunoassay	ANOVA	$p < 0.05$	Whole blood collected, processed and frozen in < 1 h, thawed and immediately exposed to experimental condition and tested
Aziz [23]	3 HIV seropositive patients and 4 seronegative patients for freeze/thaw testing, 6 HIV seropositive patients and 5 seronegative patients for storage testing	ELISA	Pearson correlation coefficients	$p < 0.01$	Whole blood collected and processed within 1–3 h, frozen, thawed and immediately exposed to experimental condition and tested
Aziz [24]	16 healthy volunteers	ELISA	Generalized estimating equations	$p < 0.05$	Whole blood collected, exposed to experimental condition, processed and frozen in < 30 min, thawed once, and immediately tested
Brøndum [25]	86 patients undergoing radiotherapy for head and neck cancers, 33 healthy controls	Bead-based multiplex immunoassay	Paired *t*-test	$p < 0.05$	Whole blood collected and processed in < 3 h, frozen for one week or more, thawed on ice, exposed to experimental condition, then immediately tested
Chaigneau [26]	2 healthy volunteers	ELISA	% change from time 0	% change < 5%	Whole blood collected, processed, and frozen, thawed at 37C, exposed to experimental condition, then tested
De Jager 2009 [13]	4 healthy pediatric volunteers	Bead-based multiplex immunoassay	unclear	$p < 0.05$	Whole blood collected, processed, and frozen in < 1 h, thawed, incubated for 1 h at RT, stimulated with LPS and PHA, exposed to experimental condition, and tested
Flower [27]	22 healthy volunteers	ELISA	Mann-Whitney	$p < 0.05$	Whole blood collected, processed, exposed to experimental condition, and frozen, then thawed at RT and immediately tested
Fraser [20]	6 healthy quality control samples, 6 quality control samples with ulcerative colitis, 6 clinical trial participants	Bead-based multiplex immunoassay	% recovery	% recovery ± 30% from baseline	Serum samples from a biobank were thawed, spiked with quality control cytokine samples, exposed to experimental condition, and immediately tested
Friebe 2008 [28]	24 patients with sepsis in ICU	Electrochemi-luminescence assay	repeated measures ANOVA	$p < 0.05$	Whole blood collected, processed, exposed to experimental condition, and immediately tested
Graham [29]	30 healthy pediatric volunteers and 110 healthy parents	Electrochemi-luminescence assay	Wilcoxon Signed Rank	$p < 0.05$	Whole blood collected and processed within 24 h, frozen, thawed on ice, exposed to experimental condition, and tested within 24 h
Guo [30]	9 healthy volunteers	Chip-based multiplex immunoassay	ANOVA	$p < 0.05$	Whole blood collected, kept at RT for 30 min, processed, exposed to experimental condition, and tested immediately
Hennø [14]	10 healthy volunteers	Bead-based multiplex immunoassay	ANOVA	$p < 0.05$	Whole blood collected, processed, exposed to experimental condition, frozen, thawed on ice, held on ice for one h, then tested
Hosnijeh [31]	10 healthy volunteers	Bead-based multiplex immunoassay	Spearman's rank correlation coefficients	$p < 0.05$	Whole blood collected and processed in < 2 h, exposed to experimental condition, and tested immediately
Huang [32]	55 healthy volunteers	Bead-based multiplex immunoassay	Wilcoxon Signed Rank	$p < 0.05$	Whole blood collected, processed, and frozen in < 2 h (frozen for 14–21 years), thawed at 4C, exposed to experimental condition, and tested
Kenis [33]	5 post-surgical patients in ICU, 3 healthy volunteers	ELISA	Student *t*-test	$p < 0.05$	Whole blood collected, processed, and exposed to experimental condition, frozen at −20C, thawed at RT, and tested immediately
Peck Palmer [34]	16 patients with severe sepsis in ICU, 10 healthy volunteers	Bead-based multiplex immunoassay	Wilcoxon Signed Rank	$p < 0.05$	Whole blood collected, processed, immediately exposed to experimental condition, frozen, thawed, and tested
Parkitny [35]	19 patients with hand or wrist fracture in the last 7–14 days	Bead-based multiplex immunoassay	Intraclass correlation coefficients (ICC) with 95% confidence intervals based on a two-way mixed-effects model, Bland-Altman analysis	ICC < 0.75	Whole blood collected, processed, and frozen, thawed at RT, held at RT for one hour, exposed to experimental condition, and tested
Ray [18]	Recombinant standards in heat-treated charcoal stripped serum reconstituted in serum from a healthy volunteer	Bead-based multiplex immunoassay	% recovery	% recovery ± 20% of controls	Recombinant cytokine standards were spiked in heat-treated charcoal stripped serum, processed, reconstituted, frozen, thawed, exposed to experimental condition, and tested
Skogstrand [36]	5 healthy volunteers	Bead-based multiplex immunoassay	Wilcoxon Signed Rank	$p < 0.0625$	Whole blood collected, processed, and frozen at −20C, thawed, exposed to experimental condition, and tested

(*continued on next page*)

Table 2 (*continued*)

First author and year	Population and sample size	Assay	Statistical tool used in analyses	Statistical significance	Comments
Thavasu [19]	63 healthy volunteers, spiked with recombinant standards	Immuno-radiometric assay	ANOVA	$p < 0.05$	Whole blood collected, processed, spiked with recombinant cytokines, exposed to experimental condition, and tested
van Waateringe [37]	82 healthy volunteers, 75 patients with diabetes, 83 patients with a history of myocardial infarction, 80 obese patients enrolled in a weight-reduction program	ELISA	Passing-Bablok correlation, Bland-Altman analysis	unclear	Whole blood collected, kept at 4C for 90 min, processed, frozen, thawed, aliquoted, re-frozen, thawed, exposed to experimental condition, and tested
Vincent [14]	10 patients with systemic lupus erythematosus	Chip-based multiplex immunoassay	Paired *t*-test and Wilcoxon Signed Rank	$p < 0.05$	Whole blood collected, processed, exposed to experimental condition, frozen, thawed, and tested
Zander [38]	5 pooled blood aliquots from critically ill patients and 5 pooled blood aliquots from outpatients	Bead-based multiplex immunoassay	Coefficient of Variation and Reference Change Values	$p < 0.01$	Serum and citrate samples were pooled, processed, exposed to experimental condition, and tested

differences between assays. Enzyme-linked immunosorbent assays (ELISA) are historically used for cytokine analysis, and are known to have a high sensitivity and specificity. Multiplex assays allow detection of multiple analytes simultaneously, which can broaden researchers' understanding of cytokine signaling cascades. There are bead-based multiplex immunoassays that rely on fluorescence reporting such as a cytometric bead assay (CBA) or Flometrix produced by Luminex [10], and plate-based immunoassays such as the Meso Scale Discovery electrochemiluminescence [11]. One of the reasons for a lack of inter-assay agreement is the presence of endogenous plasma proteins including heterophilic antibodies, soluble receptors, and complement which can interfere with test findings [12,13].

Method of blood collection itself can impact results. Blood samples collected in ethylenediaminetetraacetic acid (EDTA), heparin, or sodium citrate-containing tubes compared to those without additive may yield varying levels of cytokines. When cytokines are measured in the same patient, in blood samples collected at the same time and handled in the same way, serum levels of cytokines are usually lower in those containing anticoagulant samples [14,15]– this is believed to be due to the presence of immunothrombosis, a theory that the introduction of thrombus incites an immunologic response [16,17].

Source of cytokines – recombinant or endogenous – has been shown to effect results. Three papers used blood samples spiked with cytokines [18–20], with one of these papers comparing storage stability of spiked cytokines versus endogenous cytokines [20]. This paper did find that levels of spiked recombinant samples reacted differently than endogenous cytokines, and that endogenous cytokines are actually more stable. One reviewed study from healthy volunteers has also looked into cytokine measurement reliability when samples were collected in "sterile and non-pyrogenic" conditions (ie, endotoxin free containers) versus following "normal" blood collection procedures, to be assured that using a non-endotoxin free container did not incite cytokine release [19]. Of the 6 cytokines evaluated, the only difference that was identified was IFNɣ concentration was significantly reduced when collected under sterile non-pyrogenic procedures but was not reduced when collected normally and stored under identical conditions. Differences in sterile versus normal blood collection conditions and relevance to cytokine measurements will not be further addressed in this paper.

Little is known about the optimal sample storage conditions for future measurements of cytokines, which like other blood proteins can degrade, or be released from cells after sample collection. When stored as whole blood, this release can happen in less than 2 h [21]. Cytokines themselves form a complex cascading communication network, and it is presumed that the presence of cytokines in the blood sample when taken from the patient could lead to the production and fluctuation in levels of other cytokines in the cascade [1]. Addressing the stability of various cytokines when stored at various temperatures or exposed to repetitive freeze/thaw cycles is the focus of the remainder of this review.

2. Methods

Our study is not intended as an exhaustive review of all cytokines. Studies were identified to be included in this analysis by searching PubMed for "cytokine AND stability AND human AND (temperature OR storage) AND (blood OR plasma OR serum)". This yielded 191 results stretching back to 1971. Twenty-three studies were found to include cytokines and the experimental treatment of undergoing freeze/thaw cycles or extended storage at specific temperatures with measurements of cytokine concentrations before and after the treatment and this constituted the final sample for the focus of this paper wherein effect of storage temperatures and number of sample freeze/thaw cycles on measured concentrations of cytokines were assessed (Fig. 2).

Heterogeneity in the studied populations, targeted cytokines, analytic platform and statistical methodologies were noted and are summarized in Table 2.

Table 3.1a
IL-1α storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	1 h	sig	Thavasu et al. [19]
		4C	10 h	NS	
		4C	30 days	NS	Vincent et al. [15]
Plasma	Heparin	−80C	36 months	sig	De Jager et al. [13]
		4C	6 days	NS	Guo et al. [30]
		RT	1 h	sig	Thavasu et al. [19]
		4C	10 h	NS	
Serum	None	4C	6 days	NS	Guo et al. [30]
		RT	1 h	sig	Thavasu et al. [19]
		4C	10 h	NS	
		4C	30 days	NS	Vincent et al. [15]

Table 3.1b
IL-1α freeze/thaw stability.

Sample type	Anticoagulant	# of Freeze/Thaw Cycles	Statistically significant?	Source
Plasma	Citrate	3	NS	Hosnijieh et al. [31]
Plasma	EDTA	6	NS	Thavasu et al. [19]
Plasma	Heparin	1	sig	De Jager et al. [13]
		10	NS	Guo et al. [30]
		6	NS	Thavasu et al. [19]
Serum	None	10	NS	Guo et al. [30]
		6	NS	Thavasu et al. [19]

When looking at the results of these studies below, it is important to keep in mind that the studies are highly variable in the number of patients analyzed, the type of patients recruited, sample handling, and the robustness of statistical analysis. Also important to note is the studies that use recombinant cytokines spiked into human blood as the recombinant cytokines may react differently from those produced naturally by the immune system [18–20]. The results of the studies using spiked recombinant cytokines are discussed at the end of the paper.

3. The Interleukin-1 receptor cytokine family

Interleukin-1α, also known as IL-1F1, is a cytokine with proinflammatory properties that is part of the IL-1 system. IL-1α, IL-1β (or IL-1F2), and IL-18 (IL-1F4) are agonists in the system, and interleukin-1 receptor antagonist or IL-1RA (or IL-1F3) is an antagonist in the system [39]. Endotoxins from Gram-negative bacteria, exotoxins from Gram-positive bacteria, T cells, tumor necrosis factor (TNF), IL-2, and lipopolysaccharides complexed with interferon gamma (IFNγ) or granulocyte-macrophage colony-stimulating factor (GM-CSF) can induce IL-1 transcription. IL-4, IL-10, IL-13 and glucocorticoids suppress IL-1 production. IL-1RA is produced by monocytes, macrophages, polymorphonuclear neutrophils, and fibroblasts [39].

3.1. Interleukin-1α

IL-1α, when refrigerated as serum, or following plasma separation using EDTA or heparin, appears stable for 6–30 days [15,19,30] (Table 3.1a). Samples should not be left at room temperature for even an hour prior to IL-1α quantification [19]. Additionally, all studies except De Jager et al. [13] find IL-1α stable after 3–10 freeze/thaw cycles in a variety of media [19,30,31] (Table 3.1b).

3.2. Interleukin-1β

Like IL-1α, IL-1β appears stable at 4C following plasma isolation using citrate for 4 h, using EDTA for 48 h, or using heparin or as serum for 6 days [14,19,30,36] (Table 3.2a). Samples should not be left at

Table 3.2a
IL-1β storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
		35C	4 h	sig	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	48 h	NS	
		RT	10 h	NS	Thavasu et al. [19]
		4C	10 h	NS	
Plasma	Heparin	−80C	36 months	sig	De Jager et al. [13]
		4C	6 days	NS	Guo et al. [30]
		RT	10 h	NS	Thavasu et al. [19]
		4C	10 h	NS	
Serum	None	4C	6 days	NS	Guo et al. [30]
		RT	10 h	NS	Thavasu et al. [19]
		4C	10 h	NS	

Table 3.2b
IL-1β freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
		1	sig	Hosnijieh et al. [31]
Plasma	EDTA	3	NS	Hennø et al. [14]
		6	sig	
		1	NS	Parkitny et al. [35]
		6	NS	Thavasu et al. [19]
Plasma	Heparin	1	sig	De Jager et al. [13]
		1	sig	Guo et al. [30]
		6	NS	Thavasu et al. [19]
Serum	None	1	sig	Guo et al. [30]
		1	sig	Parkitny et al. [35]
		2	NS	Ray et al. [18]
		6	NS	Thavasu et al. [19]

room temperature prior to quantification of IL-1β – most studies found significant changes after just 4 h [14,19,22,36]. The evidence on freeze/thaw stability is a little more diverse. It would appear frozen storage of samples following plasma isolation in EDTA are least susceptible to significant changes [14,18,19,31,35] (Table 3.2b).

Table 3.3a
IL-1RA storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	24 h	sig	Aziz et al. [24]
		4C	24 h	sig	
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
Plasma	Heparin	RT	24 h	sig	Aziz et al. [24]
		4C	24 h	sig	
Serum	None	RT	24 h	NS	Aziz et al. [24]
		4C	24 h	sig	
		4C	30 days	NS	Vincent et al. [15]

3.3. Interleukin-1RA

The results when looking at storage stability of IL-1RA at different temperatures overall shows IL-1RA to be fairly unstable, with significant differences shown in every combination of anticoagulant and storage temperature except plasma isolated with citrate refrigerated and serum at room temperature [14,15,24] (Table 3.3a). IL-1RA appears stable when quantified after freeze-thaw cycles in all media, although Huang et al. [32] found a significant difference after 2 freeze-thaw cycles for samples stored as sera [14,29,32] (Table 3.3b). More research needs to be done as no anticoagulant has contesting or confirmatory data.

3.4. Interleukin-18

Interleukin-18 or IL-1F4 is a proinflammatory cytokine that is an

Table 3.3b
IL-1RA freeze/thaw stability.

Sample type	Anticoagulant	# of Freeze/Thaw Cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
Plasma	EDTA	6	NS	Hennø et al. [14]
Plasma	Heparin	5	NS	Graham et al. [29]
Serum	None	5	NS	Graham et al. [29]
		2	sig	Huang et al. [32]

Table 3.4a
IL-18 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	35C	4 h	sig	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	48 h	NS	
		4C	30 days	NS	Vincent et al. [15]
Plasma	Heparin	−80C	36 months	NS	De Jager et al. [13]
Serum	None	4C	30 days	NS	Vincent et al. [15]

Table 3.4b
IL-18 freeze/thaw stability.

Sample type	Anticoagulant	# of Freeze/thaw cycles	Statistically significant?	Source
Plasma	Heparin	1	sig	De Jager et al. [13]
		5	NS	Graham et al. [29]
Serum	None	5	NS	Graham et al. [29]

Table 4.1a
IL-6 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
		RT	24 h	NS	Peck Palmer et al. [34]
		4C	24 h	NS	
		−80C	24 h	NS	
Plasma	EDTA	RT	6 h	sig	Aguilar-Mahecha et al. [22]
		RT	24 h	NS	Aziz et al. [24]
		4C	24 h	NS	
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
		RT	24 h	NS	Peck Palmer et al. [34]
		4C	24 h	NS	
		−80C	24 h	NS	
		35C	48 h	NS	Skogstrand et al. [36]
		RT	24 h	sig	
		4C	48 h	NS	
		RT	1 h	sig	Thavasu et al. [19]
		4C	1 h	sig	
		−80C	4 years	NS	van Waateringe et al. [37]
		4C	30 days	NS	Vincent et al. [15]
Plasma	Heparin	RT	24 h	sig	Aziz et al. [24]
		4C	24 h	NS	
		RT	8 h	NS	Friebe et al. [28]
		4C	24 h	NS	
		−20C	24 h	NS	
		−80C	24 h	NS	
		4C	6 days	NS	Guo et al. [30]
		−80C	36 months	sig	De Jager et al. [13]
		RT	24 h	NS	Peck Palmer et al. [34]
		4C	24 h	NS	
		−80C	24 h	NS	
		RT	1 h	sig	Thavasu et al. [19]
		4C	1 h	sig	
Serum	None	RT	24 h	NS	Aziz et al. [24]
		4C	24 h	NS	
		RT	4 h	sig	Flower et al. [27]
		4C	4 h	NS	
		RT	8 h	NS	Friebe et al. [28]
		4C	24 h	NS	
		−20C	24 h	NS	
		−80C	24 h	NS	
		4C	6 days	NS	Guo et al. [30]
		40C	11 days	sig	Kenis et al. [33]
		30C	21 days	NS	
		20C	21 days	NS	
		4C	21 days	NS	
		−20C	21 days	NS	
		4C	30 days	NS	Vincent et al. [15]

agonist in the IL-1 system. It is part of a pathway leading to cell damage by release of nitric oxide, reactive oxygen species, and TNFα [40]. It plays an important role in defending against intracellular and extracellular microbes and is thought to help defend against viral infections as well.

Plasma separated with EDTA or heparin or stored as serum, at 4C is stable for up to 30 days when quantifying IL-18 concentrations [13,15,36] (Table 3.4a). De Jager, et al. [13] reports a difference in concentration after only one freeze-thaw cycle in heparin, whereas Graham et al. [29] finds IL-18 readings similar in up to 5 freeze-thaw cycles in heparin and sera (Table 3.4b). De Jager et al. [13] has a smaller sample size of 4 patients and uses samples stimulated with LPS, while Graham et al. [29] has 140 patients and did not stimulate their samples.

4. The Class I/hematopoietin receptor family of cytokines

The Class I or hematopoietin receptor family of cytokines all bind to receptors that share similarities in their extracellular domains [41]. This receptor family can be broken down into subfamilies based on an element of their heterodimeric or heterotrimeric receptors. IL-6 uses a gp130 chain to bind; GM-CSF and IL-5 use a common β chain; IL-2, IL-4, IL-7, IL-9, and IL-15 use a common ɣ chain, and IL-13 uses an α chain. IL-12 and G-CSF do not belong to a subfamily but are classified by their hematopoietin receptor.

4.1. Interleukin-6

The most well-studied cytokine to date is interleukin-6 (IL-6), perhaps due to early recognition that this cytokine is upregulated in most pathological states in humans. It has been implicated in sepsis, cancers, inflammatory bowel conditions, and bone disease [7].

It appears that in all media tested, IL-6 remains stable at temperatures at 4C and below for perhaps up to 30 days [14,15,24,34,36,37] with the exception of the findings by de Jager et al. [13] that storage at −80C for 36 months results in a significant change from baseline (Table 4.1a). De Jager's controls were samples that were quantified 3 years earlier, possibly resulting in variability of their immunoassay system.

Freeze/thaw cycles' effects on IL-6 are more varied (Table 4.1b). Parkitny et al. [35]) used an unusual statistical test, the intraclass correlation coefficient (ICC) and their 95% confidence intervals based on a two-way mixed-effects model and a Bland-Altman analysis. This is a measure of reliability moreso than a measure of correlation, and goes along with their explanation that they are testing the reliability of the multiplex array system in addition to the experimental treatment of freeze/thaw cycles [35]. Therefore, putting Parkitny's results aside for plasma isolated with EDTA or samples stored as serum, it would appear freezing and thawing samples up to ten times before quantifying IL-6 is not likely to affect IL-6 concentration [13,14,18,25,27,29,30,33]. Interestingly, in the citrate sample group, Hosnijieh et al. [31] and Hennø et al. [14]; use the same experimental sample size of 10 healthy volunteers but analyze the data differently. Hennø compares his experimental group with a totally different 182-person control group using ANOVA and Hosnijieh compares his samples in a before and after method with Spearman's rank correlation coefficients. In general, in plasma separated with EDTA or heparin, or samples stored as serum, it is probably safe to expose a sample to up to 3 and possibly as many as 6–10 freeze/thaw cycles prior to inducing any significant changes in IL-6 concentration.

4.2. Granulocyte-macrophage colony-stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) augments the activity of phagocytes like neutrophils, macrophages, dendritic cells, and eosinophils [42].

No one except Skogstrand et al. [36] has researched storage stability of GM-CSF but this group's findings would make it appear that plasma stored in EDTA tubes could spend 4 h at room temperature prior to quantifying GM-CSF and be accurate (Table 4.2).

4.3. Interleukin-5

Interleukin-5 (IL-5) is produced by T-cells and has an important role in eosinophil response to allergens and parasites [43].

There is no agreement in findings for IL-5 stability in storage or after freeze-thaw cycles. At this point, more research should be done, and

Table 4.1b
Freeze/thaw stability of IL-6.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
		1	sig	Hosnijieh et al. [31]
Plasma	EDTA	6	NS	Flower et al. [27]
		6	NS	Hennø et al. [14]
		1	sig	Parkitny et al. [35]
		3	NS	Thavasu et al. [19]
Plasma	Heparin	4	NS	Brøndum et al. [25]
		5	NS	De Jager et al. [13]
		5	NS	Graham et al. [29]
		10	NS	Guo et al. [30]
		1	sig	Thavasu et al. [19]
Serum	None	5	NS	Graham et al. [29]
		10	NS	Guo et al. [30]
		4	NS	Kenis et al. [33]
		1	sig	Parkitny et al. [35]
		4	NS	Ray et al. [18]
		3	NS	Thavasu et al. [19]

Table 4.2
GM-CSF Storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	35C	48 h	NS	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	4 h	sig	

clinical or research applications relying on IL-5 concentrations should quantify IL-5 expeditiously [13,14,22,31,35,36] (Tables 4.3a and 4.3b).

4.4. Interleukin-2

Interleukin-2 (IL-2) is mainly produced by CD4+ T cells that have been activated by an antigen [44]. IL-2 amplifies the T-cell immune response and can increase the proliferation of both cytotoxic and suppressor T-cells.

The existing data shows IL-2 is stable when left at room temperature in plasma isolated using EDTA for up to 6 h, or when refrigerated using heparin or as serum for at least 6 days [13,22,30] (Table 4.4a). When quantified after freeze/thaw cycles, only De Jager et al. [13] shows a difference after 1 cycle (Table 4.4b). Brøndum et al. [25] and Guo et al. [30] both show stability of IL-2 levels in heparin after at least 4 freeze/thaw cycles, which represent 128 patient samples as opposed to de Jager's 4.

4.5. Interleukin-4

Interleukin 4 (IL-4) is important for the adaptive immune response by increasing B cell surface major histocompatibility complex antigens and stimulating growth of helper and cytotoxic T cells [45]. It is secreted by T cells, natural killer T cells, basophils, eosinophils, and mast cells.

The majority of evidence shows that IL-4 is stable when refrigerated in plasma separated by EDTA for at least 2 days, and separated by heparin or as serum for up to 6 days [14,30,36] (Table 4.5a). Citrate storage produces less reliable data based on current studies [14]. As for freeze/thaw cycles, the results show a fairly unstable cytokine with significant changes after just 1 freeze/thaw cycle except in plasma isolated by citrate [13,25,30,31,35] (Table 4.5b). Interestingly, De Jager et al. [13] found that 1 freeze/thaw cycle of plasma separated by heparin produced significant change, but when stored at −80C in heparin for three years, then quantified, the changes in IL-4 concentration were not significant. In general, it seems quantifying IL-4 before freezing samples would be advisable.

Table 4.3a
IL-5 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
		35C	48 h	NS	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	4 h	sig	
Plasma	Heparin	−80C	36 months	sig	De Jager et al. [13]

Table 4.3b
IL-5 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw Cycles	Statistically significant?	Source
Plasma	Citrate	3	NS	Hosnijieh et al. [31]
Plasma	EDTA	1	sig	Parkitny et al. [35]
Plasma	Heparin	1	sig	De Jager et al. [13]
Serum	None	1	sig	Parkitny et al. [35]

Table 4.4a
IL-2 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
Plasma	Heparin	−80C	36 months	NS	De Jager et al. [13]
		4C	6 days	NS	Guo et al. [30]
Serum	None	4C	6 days	NS	Guo et al. [30]

Table 4.4b
IL-2 freeze/thaw stability.

Sample type	Anticoagulant	# of Freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	3	NS	Hosnijieh et al. [31]
Plasma	Heparin	4	NS	Brøndum et al. [25]
		1	sig	De Jager et al. [13]
		10	NS	Guo et al. [30]
Serum	None	10	NS	Guo et al. [30]

4.6. Interleukin-7

Interleukin-7 (IL-7) is a non-redundant signaler for T-cell and B-cell differentiation from hematopoietic stem cells [46].

The results show IL-7 quantification is stable when samples are stored at 4C, though IL-7 may remain stable in plasma isolated by citrate at room temperature for 4 h [14,22] (Table 4.6a). More research needs to be done on stability after freeze/thaw cycles, currently freezing IL-7 prior to quantification should not be done (Table 4.6b).

4.7. Interleukin-9

Interleukin-9 (IL-9) plays an important role in asthma and in parasite response, signaling mast cell proliferation in the bone marrow [47]. IL-9 also specifically promotes mucin production in the lung epithelium.

Hennø et al. [14] and Aguilar-Mahecha et al. [22] agree that IL-9 is stable even at room temperature for up to 6 h in plasma when isolated by EDTA (Table 4.7a). Hennø provides the only published reports on IL-9 freeze-thaw cycles [14] (Table 4.7b). In all conditions studied, IL-9 appears stable.

4.8. Interleukin-15

Interleukin-15 (IL-15) helps protect against viral and bacterial infections by stimulating NK cells, T cells, and B cells to proliferate and secrete other cytokines; and by stimulating phagocytes [48] HYPERLINK "SPS:refid::bib48" .

More research needs to be done on storage stability and freeze-thaw cycles (Tables 4.8a and 4.8b).

4.9. Interleukin-13

Interleukin-13 (IL-13) is produced by T cells, NK cells, basophils, eosinophils, mast cells, dendritic cells, and lymphoma tumor cells [49]. It is important for allergic responses, healing and protection from helminth infections, and asthmatic responses.

It would appear IL-13 is stable if refrigerated for at least 4 h as

Table 4.5a
IL-4 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	sig	Hennø et al. [14]
		4C	4 h	sig	
Plasma	EDTA	RT	6 h	sig	Aguilar-Mahecha et al. [22]
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
		35C	48 h	NS	Skogstrand et al. [36]
		RT	48 h	NS	
		4C	48 h	NS	
Plasma	Heparin	−80C	36 months	NS	De Jager et al. [13]
		4C	6 days	NS	Guo et al. [30]
Serum	None	4C	6 days	NS	Guo et al. [30]

Table 4.5b
IL-4 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	3	NS	Hosnijieh et al. [31]
Plasma	EDTA	1	sig	Parkitny et al. [35]
Plasma	Heparin	4	NS	Brøndum et al. [25]
		1	sig	De Jager et al. [13]
		1	sig	Guo et al. [30]
Serum	None	1	sig	Guo et al. [30]
		1	sig	Parkitny et al. [35]

Table 4.6a
IL-7 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	

Table 4.6b
IL-7 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw Cycles	Statistically significant?	Source
Plasma	EDTA	1	sig	Parkitny et al. [35]
Serum	None	1	sig	Parkitny et al. [35]

plasma separated with citrate or EDTA [14,22] (Table 4.9a). Until further study is completed, storage of IL-13 as plasma separated with heparin is not recommended [13]. For freeze/thaw cycles, it would be recommended that samples to be quantified for IL-13 be stored as serum based on Parkitny et al.'s [35] (Table 4.9b) and Fraser et al.'s [20] findings (Table 4.9a).

4.10. Interleukin-12

Interleukin-12 (IL-12) is a regulator of immune response, predominantly cell-mediated immunity [50]. It consists of two subunits: p40 and p35. The p40 subunit is only expressed by antigen-presenting cells such as monocytes, macrophages, and dendritic cells. The p35 subunit is expressed by many types of cells, but its production is tightly regulated in antigen-presenting cells, thus controlling the amount of IL-12 secreted.

Aguilar-Mahecha et al. [22], Aziz et al. [24], and Skogstrand et al. [36] review the temperature storage stability of IL-12 (Table 4.10a). When isolated by EDTA, plasma can be stored refrigerated for up to 48 h prior to quantifying IL-12 concentration [22,24,36]. When isolated by heparin or stored as sera, further analysis is needed by other groups, but Aziz et al.'s [24] findings suggest in heparin it is safe to store for up to 24 h at room temperature or refrigerated prior to quantification [24]. As for freeze/thaw stability, only Hosnijieh et al. [31] showed comparable concentration of IL-12 after up to 3 freeze/thaw studies when isolated in plasma by citrate [13,31,35] (Table 4.10b).

4.11. Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (G-CSF) acts to produce

Table 4.7a
IL-9 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	

Table 4.7b
IL-9 freeze/thaw cycle stability.

Sample type	Anticoagulant	# of Freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
Plasma	EDTA	6	NS	Hennø et al. [14]

Table 4.8a
IL-15 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
Plasma	Heparin	−80C	12 months	sig	De Jager et al. [13]

Table 4.8b
IL-15 freeze/thaw cycle stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Serum	None	1	sig	De Jager et al. [13]

Table 4.9a
IL-13 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
Plasma	Heparin	−80C	12 months	sig	De Jager et al. [13]
Serum	None	−70C [QC]	4 months	NS	Fraser
		−70C [QC]	5 months	sig	
		−70C	15 months	NS	

*QC = "quality control" recombinant cytokine spiked samples

Table 4.9b
IL-13 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	1	sig	Hosnijieh et al. [31]
Plasma	EDTA	1	sig	Parkitny et al. [35]
Plasma	Heparin	4	NS	Brøndum et al. [25]
		1	sig	De Jager et al. [13]
Serum	None	1	NS	Parkitny et al. [35]

Table 4.10a
IL-12 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	6 h	sig	Aguilar-Mahecha et al. [22]
		RT	24 h	NS	Aziz et al.
		4C	24 h	NS	[24]
		35C	48 h	NS	Skogstrand
		RT	24 h	NS	et al. [36]
		RT	48 h	sig	
		4C	48 h	NS	
Plasma	Heparin	RT	24 h	NS	Aziz et al.
		4C	24 h	NS	[24]
Serum	None	RT	24 h	NS	Aziz et al.
		4C	24 h	sig	[24]

neutrophils both at a basal level and in response to infection [51]. It is produced by bone marrow stromal cells, fibroblasts, endothelial cells, and activated macrophages.

The results here suggest G-CSF is better stored refrigerated or colder for any length of time [14,22] (Table 4.11a). Parkitny et al. [35] found significant differences after 1 freeze/thaw cycle when quantifying G-CSF in plasma separated with EDTA or serum, while other researchers found 3–4 freeze thaw cycles has no effect on concentration [25,32] (Table 4.11b). Parkitny uses intraclass correlation coefficients, whereas Brøndum et al. [25] uses t-tests and Huang et al. [32] uses Wilcoxon Rank Sums to analyze data.

5. Class II cytokine receptor family

The Class II receptor family of cytokines includes interleukin-10 and interferon cytokines. Like the Class I Receptor Family, this family binds to receptors that share structural similarities in their extracellular domains.

5.1. Interferon α

Interferon alpha (IFNα) is a type I interferon, which play important roles in responding to viral infection, intracellular bacterial infections, and endotoxin expressed by Gram-negative bacteria [52]. IFNα is mainly secreted by lymphocytes.

Table 4.10b
IL-12 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	3	NS	Hosnijieh et al. [31]
Plasma	EDTA	1	sig	Parkitny et al. [35]
Plasma	Heparin	1	sig	De Jager et al. [13]
Serum	None	1	sig	Parkitny et al. [35]

Table 4.11a
G-CSF storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	6 h	sig	Aguilar-Mahecha et al. [22]
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	

Table 4.11b
G-CSF freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	EDTA	1	sig	Parkitny et al. [35]
Plasma	Heparin	4	NS	Brøndum et al. [25]
Serum	None	3	NS	Huang et al. [32]
		1	sig	Parkitny et al. [35]

Table 5.1
IFNα storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	1 h	sig	Thavasu et al. [19]
		4C	10 h	NS	
Plasma	Heparin	RT	1 h	sig	Thavasu et al. [19]
		4C	10 h	NS	
Serum	None	RT	1 h	sig	Thavasu et al. [19]
		4C	10 h	NS	

Table 5.2a
IFNɣ storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	6 h	sig	Aguilar-Mahecha et al. [22]
		RT	24 h	NS	Aziz et al. [24]
		4C	24 h	NS	
		RT	20 days	NS	Aziz et al. [23]
		4C	20 days	NS	
		−70C	20 days	NS	
		35C	48 h	NS	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	48 h	NS	
		RT	1 h	sig	Thavasu et al. [19]
		4C	1 h	sig	
Plasma	Heparin	RT	24 h	NS	Aziz et al. [24]
		4C	24 h	NS	
		4C	6 days	NS	Guo et al. [30]
		RT	1 h	sig	Thavasu et al. [19]
		4C	1 h	sig	
Serum	None	RT	24 h	NS	Aziz et al. [24]
		4C	24 h	NS	
		RT	20 days	NS	Aziz et al. [23]
		4C	20 days	NS	
		−70C	20 days	NS	
		4C	6 days	NS	Guo et al. [30]
		RT	1 h	sig	Thavasu et al. [19]
		4C	1 h	sig	
		4C	30 days	NS	Vincent et al. [15]

More studies need to be done to make recommendations regarding stability of IFNα (Table 5.1). To date, no studies have examined the effect of freeze/thaw on IFNα concentrations.

5.2. Interferon ɣ

Interferon gamma is produced by T lymphocytes and natural killer cells in response to immune and inflammatory stimuli [53]. It is one of the most well-studied cytokines and has roles in antiviral activity, macrophage activation, innate immune responses, adaptive immune responses (via increasing the number of major histocompatibility complexes on cell surfaces for antigen presentation), T cell development, humoral immunity, tumor immunity, and antiproliferation [53].

IFNɣ storage stability has been relatively well-studied (Table 5.2a). IFNɣ appears stable when stored as plasma separated with EDTA or as serum at 4C or colder for up to 30 days [15,19,22,24,30].

Besides Parkitny et al. [35] and De Jager et al. [13], when quantifying IFNɣ, concentrations are accurate for at least 6 freeze-thaw cycles if stored as plasma isolated by citrate and for at least 10 freeze thaw cycles if isolated by EDTA or heparin, or stored as sera [14,23,30] (Table 5.2b). Parkitny uses intraclass correlation coefficients for analysis which may explain the difference in significant findings.

5.3. Interleukin-10

Interleukin-10 (IL-10) has immunosuppressant capabilities, turning off cytokine production by T cells, monocytes, macrophages, and dendritic cells [54]. IL-10 is expressed by activated T cells, B cells, macrophages and monocytes, NK cells, keratinocytes, eosinophils, mesangial cells, epithelial cells, and tumor cells [54]. Its production is induced by a variety of pathogens. In human disease, IL-10 has been shown to improve inflammatory bowel disease [54].

For storage, Kenis et al. [33] and Guo et al. [30] agree that serum samples should be kept at 4C if future quantification of IL-10 is desired (Table 5.3a). IL-10 appears more stable when stored as plasma separated with EDTA, with various reports showing stability even at room temperature [22,36]. De Jager et al.'s [13] finding of a significant difference after 3 years of storage at −80C in heparin may be related to the elapsed time and potential introduction of bias [13].

With the exception of Guo et al. [30], all researchers have found that freeze/thaw cycles do not affect the concentration of IL-10 when it is stored as plasma isolated by citrate (up to 3 thaws), heparin (up to 5 thaws), and when stored as serum (up to 5 thaws) [13,18,33,30,31] (Table 5.3b). Graham et al. [29] studied 140 patients compared to Guo's 9.

6. Tumor necrosis factor receptor family

The tumor necrosis factor (TNF) receptor family of cytokines are so grouped together because the receptors all contain intracellular "death" domains [41].

6.1. Tumor necrosis factor α

Tumor necrosis factor alpha (TNFα) has local effects that are important in host defense, but can be upregulated quickly and induce systemic effects such as inflammation, hypotension, coagulation, and tissue damage [55]. It is produced by monocytes in response to lipopolysaccharides, by epithelial cells in response to ultraviolet light, and in T lymphocytes after receptor activation.

The published literature here is extensive. There is agreement amongst papers using endogenous TNFα that when stored in plasma isolated by EDTA, if stored at 4C or colder, TNFα is stable for at least 20 days [23,24,28,36,37] (Table 6.1a). If stored as serum at 4C, a majority of researchers found that TNFa remained stable for up to 30 days, though possibly not 60 days [15,23,24,27,28,30,38]. When plasma was separated using heparin, there is a little more variety in the results [13,19,24,28,30]. Preferably, prior to quantification, TNFα would be stored as serum or plasma isolated by EDTA.

Table 5.2b
IFNɣ freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
		3	NS	Hosnijieh et al. [31]
Plasma	EDTA	10	NS	Aziz et al. [23]
		6	NS	Hennø et al. [14]
		1	sig	Parkitny et al. [35]
		6	NS	Thavasu et al. [19]
Plasma	Heparin	1	sig	De Jager et al. [13]
		10	NS	Guo et al. [30]
		6	NS	Thavasu et al. [19]
Serum	None	10	NS	Aziz et al. [23]
		10	NS	Guo et al. [30]
		1	sig	Parkitny et al. [35]
		6	NS	Thavasu et al. [19]

Table 5.3a
IL-10 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		35C	48 h	NS	Skogstrand et al. [36]
		RT	48 h	NS	
		4C	48 h	NS	
Plasma	Heparin	−80C	36 months	sig	De Jager et al. [13]
		4C	6 days	NS	Guo et al. [30]
Serum	None	4C	6 days	NS	Guo et al. [30]
		40C	21 days	sig	Kenis et al. [33]
		30C	21 days	sig	
		20C	21 days	sig	
		4C	21 days	NS	

Table 5.3b
IL-10 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	3	NS	Hosnijieh et al. [31]
Plasma	Heparin	5	NS	De Jager et al. [13]
		5	NS	Graham et al. [29]
		1	sig	Guo et al. [30]
Serum	None	4	NS	Kenis et al. [33]
		5	NS	Graham et al. [29]
		1	NS	Parkitny et al. [35]
		2	NS	Ray et al. [18]

TNFα is a beta-pleated sheet type molecule and is more susceptible to changes with freeze/thaw cycles [29]. This may explain, in part, the variety of findings in Table 6.1b. For samples stored in plasma separated with heparin, the only researcher to find a significant difference was De Jager et al. [13], who used cytokines whose production was stimulated by LPS, which may affect validity of results [19,25,29–30].

6.2. Tumor necrosis factor β

Tumor necrosis factor β, or lymphotoxin α, is expressed by T-lymphocytes and NK cells, and is important in lymphoid tissue development and innate and adaptive immune response cellular differentiation [56].

Skogstrand et al. [36] is uncontested, but it appears storage for 4 h or less at room temperature or refrigerated does not affect TNFβ concentrations [36] (Table 6.2). Research needs to be done looking at responsiveness to freeze/thaw cycles. TNFβ, like TNFα, is a beta-pleated sheet molecule and may be more susceptible to changes in freeze/thaw cycles [29,56].

7. Chemokine receptor family

Chemokine receptors, as stated previously, are classified by their 7 transmembrane domains. All CXC and CC chemokines are included in this family [41].

7.1. CC-motif chemokine ligand 2

CCL2 (CC-motif chemokine ligand 2) or MCP-1 (monocyte chemoattractant protein 1) chemoattracts monocytes and induces them to release enzymes [8].

Samples being quantified for CCL2 can be safely stored up to 30 days at 4C as plasma isolated by EDTA or in serum, but possibly not even 4 h at room temperature prior to quantification [14,15,30,36]

Table 6.1a
TNFα storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		RT	24 h	NS	Aziz et al. [24]
		4C	24 h	NS	
		RT	20 days	sig	Aziz et al. [23]
		4C	20 days	NS	
		−70C	20 days	NS	
		RT	8 h	NS	Friebe et al. [28]
		4C	24 h	NS	
		−20C	24 h	NS	
		−80C	24 h	NS	
		35C	48 h	NS	Skogstrand et al. [36]
		RT	24 h	sig	
		4C	48 h	NS	
		RT	1 h	sig	Thavasu et al. [19]
		4C	1 h	sig	
		−80C	4 years	NS	van Waateringe et al. [37]
Plasma	Heparin	RT	24 h	sig	Aziz et al. [24]
		4C	24 h	NS	
		−80C	36 months	sig	De Jager et al. [13]
		RT	8 h	NS	Friebe et al. [28]
		4C	24 h	NS	
		−20C	24 h	NS	
		−80C	24 h	NS	
		4C	6 days	sig	Guo et al. [30]
		RT	1 h	sig	Thavasu et al. [19]
		4C	1 h	sig	
Serum	None	RT	24 h	sig	Aziz et al. [24]
		4C	24 h	NS	
		RT	20 days	sig	Aziz et al. [23]
		4C	20 days	NS	
		−70C	20 days	NS	
		RT	4 h	sig	Flower et al. [27]
		4C	4 h	sig	
		RT	8 h	NS	Friebe et al. [28]
		4C	24 h	NS	
		−20C	24 h	NS	
		−80C	24 h	NS	
		4C	6 days	NS	Guo et al. [30]
		RT	1 h	sig	Thavasu et al. [19]
		4C	1 h	sig	
		4C	30 days	NS	Vincent et al. [15]
		4C	30 days	NS	Zander 2014
		4C	60 days	sig	
		−20C	90 days	NS	
		−80C	90 days	NS	

(Table 7.1a). Graham et al. [29] and Guo et al. [30] both show that 5 or more freeze/thaw cycles of plasma separated with heparin or stored as serum do not significantly alter CCL2 concentrations (Table 7.1b). Once again, Parkitny et al's [35] findings disagree, possibly related to the type of statistical test used that was trying to determine reliability of their immunoassay system as well as the effect of the experimental intervention [35].

7.2. CC-motif chemokine ligand 3

CC-motif chemokine ligand 3 (CCL3) or macrophage inflammatory protein 1 alpha (MIP-1α) is produced by stimulated leukocytes, fibroblasts, and tumor cells, amongst others, and acts as a chemotactic agent to monocytes, T cells, NK cells, dendritic cells, B cells, IgE-stimulated mast cells, basophils, and eosinophils. CCL3 additionally causes increased integrin expression in T cells, increased adhesions to endothelial cell walls, and T-cell activation, proliferation, and secretion of IL-2 [8].

It is unclear if storage of serum or plasma samples to later quantify CCL3 is reliable at any temperature [22,36] (Table 7.2a). When stored as plasma isolated by citrate, blood samples can be frozen and thawed up to 6 times without significant change [14] (Table 7.2b).

7.3. CC-motif chemokine ligand 4

CC-motif chemokine ligand 4 (CCL4) or macrophage inflammatory protein 1 beta (MIP-1β) is produced by stimulated leukocytes, fibroblasts, and tumor cells [8] CCL4 induces chemotaxis in T cells, monocytes, NK cells, and dendritic cells.

Blood sample storage at room temperature to later check CCL4 concentration leads to statistically significantly different results [22,24,36] (Table 7.3a). Refrigeration storage for samples stored as serum and plasma separated with heparin have no differences after 24 h [24]. EDTA-isolated plasma results vary [24,36]. One explanation is Skogstrand et al. [36] used a p-value of 0.0625 as a cutoff and was more likely to identify results as having significant differences when compared with Aziz et al's [24]cutoff of 0.05. CCL4 freeze/thaw cycles show plasma samples separated with citrate that have been frozen and thawed up to 6 times show no significant differences [14], and samples in serum thawed twice with no significant difference [32] (Table 7.3b).

7.4. CC-motif chemokine ligand 5

CC-motif chemokine ligand 5 (CCL5) or RANTES is constitutively expressed by unstimulated T cells, but is also expressed by platelets, fibroblasts, and tumor cells [8]. Like CCL3 and CCL4, it induces chemotaxis in monocytes, T cells, NK cells, and dendritic cells. CCL5 also chemoattracts basophils, IgE-stimulated mast cells and eosinophils.

CCL5 quantification after storage at room temperature or 4C in plasma separated with citrate or EDTA should be considered unreliable [14,24,36] (Table 7.4a). CCL5 when stored as serum or as plasma isolated by heparin may be able to sustain up to 24 h of refrigerated storage [24]. Measuring CCL5 after 3 freeze/thaw cycles in plasma separated with citrate or EDTA appears to produce accurate readings, however by the 6th thaw this is no longer the case [14,31] (Table 7.4b).

7.5. CC-motif chemokine ligand 11

CC-motif chemokine ligand 11 (CCL11), or eotaxin-1, is a chemoattractant for eosinophils [8].

For short periods of time (4–6 h), in plasma separated with citrate or EDTA, it is safe to keep CCL11 at room temperature or refrigerated [14,22] (Table 7.5a). Blood samples being quantified for CCL11 can be frozen and thawed at least 2 times in serum and up to 6 times in plasma isolated by citrate and EDTA [14,25,31–32] (Table 7.5b).

7.6. C-X-C motif chemokine 10

C-X-C motif chemokine 10 (CXCL10), or interferon gamma-induced protein 10 (IP-10), is produced by macrophages, T cells, fibroblasts, endothelial cells, keratinocytes, synovial cells, and tumor cells. It is constitutively expressed by thymic and splenic stromal cells [8].

CXCL10 appears to be fairly stable when stored for 4–6 h at room temperature or refrigerated in plasma isolated by citrate and EDTA [14,22], and when quantified after 3–6 freeze-thaw cycles [14,29,31] (Tables 7.6a and 7.6b).

7.7. Interleukin-8

Interleukin-8 (IL-8), or CXCL8, has the traditional two pairs of cysteines with disulfide bond structure associated with chemokines [57].

Table 6.1b
TNFα freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
		1	sig	Hosnijieh et al. [31]
Plasma	EDTA	10	NS	Aziz et al. [23]
		3	sig	Flower et al. [27]
		6	NS	Hennø et al. [14]
		1	sig	Parkitny et al. [35]
		6	NS	Thavasu et al. [19]
Plasma	Heparin	4	NS	Brøndum et al. [25]
		1	sig	De Jager et al. [13]
		5	NS	Graham et al. [29]
		10	NS	Guo et al. [30]
		6	NS	Thavasu et al. [19]
Serum	None	10	NS	Aziz et al. [23]
		5	NS	Graham et al. [29]
		10	NS	Guo et al. [30]
		3	NS	Ray et al. [18]
		1	sig	Parkitny et al. [35]
		6	NS	Thavasu et al. [19]
		2	NS	Huang et al. [32]
		3	sig	

Table 6.2
TNFβ storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	35C	48 h	NS	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	4 h	sig	

It is primarily produced by monocytes, but is also made by T cells, neutrophils, natural killer cells, endothelial cells, fibroblasts, and epithelial cells in response to proinflammatory cytokines like TNFα. It acts as a chemotactic factor for neutrophils and induces degranulation of neutrophils.

The majority of published reports show stability of IL-8 when refrigerated [14,22,28,36] (Table 7.7a). With plasma samples isolated by heparin or in serum, Guo et al. [30] found significant differences when

Table 7.1a
CCL2 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	
		35C	48 h	NS	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	48 h	NS	
		4C	30 days	NS	Vincent et al. [15]
Plasma	Heparin	4C	6 days	NS	Guo et al. [30]
Serum	None	4C	6 days	NS	Guo et al. [30]
		4C	30 days	NS	Vincent et al. [15]

Table 7.1b
CCL2 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	EDTA	1	sig	Parkitny et al. [35]
Plasma	Heparin	5	NS	Graham et al. [29]
		10	NS	Guo et al. [30]
Serum	None	5	NS	Graham et al. [29]
		10	NS	Guo et al. [30]
		1	sig	Parkitny et al. [35]

Table 7.2a
CCL3 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		35C	24 h	sig	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	48 h	sig	

Table 7.2b
CCL3 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
Plasma	EDTA	6	NS	Hennø et al. [14]
		1	sig	Parkitny et al. [35]
Serum	None	1	NS	Parkitny et al. [35]

Table 7.3a
CCL4 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	RT	6 h	sig	Aguilar-Mahecha et al. [22]
		RT	24 h	NS	Aziz et al. [24]
		4C	24 h	NS	
		35C	48 h	NS	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	24 h	sig	
Plasma	Heparin	RT	24 h	sig	Aziz et al. [24]
		4C	24 h	NS	
Serum	None	RT	24 h	sig	Aziz et al. [24]
		4C	24 h	NS	

refrigerated for 6 days, but other studies show no significant changes when refrigerated for 24 h [28,30]. De Jager et al. [13] shows a significant difference in concentration after 12 months at −80C, but as addressed previously, bias could have been introduced into the analysis system [13].

For freeze/thaw cycles, plasma separated with citrate has two studies supporting no significant change after at least 3 freeze/thaw cycles with no conflicting data [14,31] (Table 7.7b). For plasma isolated by heparin, there are 3 studies reporting no significant changes after 4 or more freeze/thaw cycles, with the only conflicting evidence being De Jager et al. [13,25,29–30]. Plasma isolated by EDTA and samples stored as serum have less consistent results, though Huang et al. [32] supports 2 freeze/thaw cycles for samples in serum as having no significant change [18,29–30,35]. If freezing samples for future quantification of IL-8, we would recommend using citrate or heparin as anticoagulants.

8. Miscellaneous ligands and their receptors

The following are a combination of secreted ligands involved in immune signaling and proper cytokines. They all have their own receptors that are not easily classified into the systems above.

8.1. Interleukin-16

Interleukin-16 (IL-16) is produced by CD8 and CD4 + T cells, mast cells, eosinophils, dendritic cells, bronchial alveolar cells, B cells, and fibroblasts; and is a chemoattractant for CD4 + cells including eosinophils, monocytes, and dendritic cells [58]. Its receptor is actually the D4 region of CD4.

IL-16 is understudied in regard to temperature stability, more research needs to be done (Table 8.1).

Table 7.4a
CCL5 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	sig	Hennø et al. [14]
		4C	4 h	sig	
Plasma	EDTA	RT	24 h	NS	Aziz et al. [24]
		4C	24 h	NS	
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
		35C	4 h	sig	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	4 h	sig	
Plasma	Heparin	RT	24 h	sig	Aziz et al. [24]
		4C	24 h	NS	
Serum	None	RT	24 h	NS	Aziz et al. [24]
		4C	24 h	NS	

Table 7.4b
CCL5 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	3	NS	Hennø et al. [14]
		6	sig	
		3	NS	Hosnijieh et al. [31]
Serum	EDTA	3	NS	Hennø et al. [14]
		6	sig	

8.2. C-reactive protein

C-reactive protein (CRP) is induced by IL-6 and their serum levels correlate in *in vivo* experiments [59]. It is important in acute phase response systems to injury or infection. It binds to phosphocholine.

CRP, when stored as plasma isolated by citrate or heparin, is shown to be stable by Peck Palmer et al. [34] for 24 h at a variety of temperatures [34] (Table 8.2a). When stored as plasma separated with EDTA, Skogstrand et al, 2008 [36] shows significant differences at just 4 h whereas Peck Palmer et al. [34] shows stability of CRP data when stored for up to 24 h at room temperature or refrigerated, which is contradictory. Both studies use bead-based multiplex immunoassays and Wilcoxon Rank Sums to analyze data, but Peck Palmer has 16 patients compared to Skogstrand's 5. For freeze/thaw cycles, Graham et al. [29] shows no significant changes in quantification of CRP after 5

Table 7.3b
CCL4 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
Plasma	EDTA	6	NS	Hennø et al. [14]
		1	sig	Parkitny et al. [35]
Serum	None	2	NS	Huang et al. [32]
		3	sig	

Table 7.5a
CCL11 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	

Table 7.5b
CCL11 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
		3	NS	Hosnijieh et al. [31]
Plasma	EDTA	6	NS	Hennø et al. [14]
Plasma	Heparin	4	NS	Brøndum et al. [25]
Serum	None	2	NS	Huang et al. [32]
		3	sig	

Table 7.6a
CXCL10 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	

Table 7.6b
CXCL10 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
		3	NS	Hosnijieh et al. [31]
Plasma	EDTA	6	NS	Hennø et al. [14]
Plasma	Heparin	5	NS	Graham et al. [29]
Serum	None	5	NS	Graham et al. [29]

cycles when stored as plasma isolated by heparin or as serum (Table 8.2b). Huang et al. [32] shows significant changes in serum after just 2 cycles. More research needs to be done on CRP freeze/thaw cycles.

8.3. Epidermal growth factor

Epidermal growth factor (EGF) is a 53-amino-acid traditional growth factor with cytokine-like actions. Its regulation and function are not well understood, but it is believed to be involved in wound healing, tissue regeneration, cytoprotection and angiogenesis, placentation, skin homeostasis, and pathological states including cancer, polycystic kidney disease, and psoriasis [60]. It binds to the epidermal growth factor receptor, which is a receptor tyrosine kinase.

Only Guo et al. [30] has analyzed storage stability of EGF and more research needs to be done to identify if EGF remains stable at any temperature for a length of time [30] (Table 8.3a). Interestingly, Guo reported that EGF concentrations in plasma isolated by heparin and serum remained stable for up to 10 freeze-thaw cycles (Table 8.3b). Huang et al. [32] confirms stability for up to 2 cycles but not 3 in serum [32].

8.4. Interleukin-17

Interleukin-17 (IL-17) is a cytokine-inducing cytokine with inflammatory and hematopoietic activities, acting on a wide variety of cell types. It has been implicated as playing a role in various autoimmune diseases, including rheumatoid arthritis, systemic lupus sclerosis, multiple sclerosis, and psoriasis [61]. It binds to its own receptor that does not have much homology with any of the other cytokine receptor families.

The results here suggest that IL-17 is not a very stable cytokine [13,35–36]. More research needs to be done to see if any length of storage at any temperature is appropriate prior to evaluating IL-17

Table 7.7a
IL-8 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	4 h	NS	Hennø et al. [14]
		4C	4 h	NS	
Plasma	EDTA	RT	6 h	NS	Aguilar-Mahecha et al. [22]
		RT	8 h	NS	Friebe et al. [28]
		4C	24 h	NS	
		−20C	24 h	NS	
		−80C	24 h	NS	
		RT	4 h	sig	Hennø et al. [14]
		4C	4 h	NS	
		35C	24 h	sig	Skogstrand et al. [36]
		RT	24 h	sig	
		4C	48 h	NS	
Plasma	Heparin	−80C	12 months	sig	De Jager et al. [13]
		RT	8 h	sig	Friebe et al. [28]
		4C	24 h	NS	
		−20C	24 h	NS	
		−80C	24 h	NS	
		4C	6 days	sig	Guo et al. [30]
Serum	None	RT	8 h	sig	Friebe et al. [28]
		4C	24 h	NS	
		−20C	24 h	NS	
		−80C	24 h	NS	
		4C	6 days	sig	Guo et al. [30]

Table 8.1
IL-16 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Serum	None	2	NS	Huang et al. [32]
		3	sig	

Table 8.2a
CRP storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Citrate	RT	24 h	NS	Peck Palmer et al. [34]
		4C	24 h	NS	
		−80C	24 h	NS	
Plasma	EDTA	35C	4 h	sig	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	4 h	sig	
		RT	24 h	NS	Peck Palmer et al. [34]
		4C	24 h	NS	
		−80C	24 h	NS	
		−80C	4 years	NS	van Waateringe et al. [37]
Serum	Heparin	RT	24 h	NS	Peck Palmer et al. [34]
		4C	24 h	NS	
		−80C	24 h	NS	

Table 7.7b
IL-8 freeze/thaw stability.

sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Citrate	6	NS	Hennø et al. [14]
		3	NS	Hosnijieh et al. [31]
Plasma	EDTA	6	NS	Hennø et al. [14]
		1	sig	Parkitny et al. [35]
Plasma	Heparin	4	NS	Brøndum et al. [25]
		1	sig	De Jager et al. [13]
		5	NS	Graham et al. [29]
		10	NS	Guo et al. [30]
Serum	None	5	NS	Graham et al. [29]
		10	NS	Guo et al. [30]
		2	NS	Huang et al. [32]
		3	sig	
		3	NS	Ray et al. [18]
		1	sig	Parkitny et al. [35]

concentrations (Table 8.4a and 8.4b).

8.5. Transforming growth factor beta 1

Transforming growth factor beta 1 (TGFβ1) can be produced by almost any cell but is mostly produced by platelets, and it is induced by several markers of stress [62]. It has important, non-redundant roles in hematopoiesis and immune cell homeostasis. Along with other members of the TGFβ family, it binds to the TGFβ receptor family.

The limited studies that have been done on TGFβ stability indicates that it should be stored at 4C or cooler for later quantification (Table 8.5a), and that it may be frozen and thawed several times (or, up to 100) [26,36] (Table 8.5b). Additional confirmatory studies would be useful.

8.6. Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is produced by macrophages, platelets, and solid tumors. It induces endothelial vascular growth and increases vascular permeability [63]. It is a major focus of research on physiologic and pathologic angiogenesis. It binds to one of three VEGF receptors.

Studies on VEGF temperature stability are dominated by Guo et al. [30]). At this point, it appears safe to store serum and plasma separated with heparin for up to 6 days at 4C [30] (Table 8.6a). There is consensus that reliable quantification occurs in plasma isolated by heparin to up to 4 and possibly 10 freeze/thaw cycles prior to quantification for VEGF [25,30]; and in serum up to 2 freeze/thaw cycles prior to quantification for VEGF [30,32] (Table 8.6b).

9. Samples spiked with recombinant cytokines

Ray et al. [18] studied freeze/thaw stability of recombinant TNFα, IL-10, IL-1β, IL-6, and IL-8 in serum. Table 3.2b shows that Ray and another recombinant cytokine study (Thavasu et al. [19] actually found IL-1β concentration unchanged after 2–6 freeze/thaw cycles, as

Table 8.2b
CRP freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Heparin	5	NS	Graham et al. [29]
Serum	None	5	NS	Graham et al. [29]
		2	sig	Huang et al. [32]

Table 8.3a
EGF storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Heparin	4C	6 days	sig	Guo et al. [30]
Serum	None	4C	6 days	sig	Guo et al. [30]

Table 8.3b
EGF freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Heparin	10	NS	Guo et al. [30]
Serum	None	10	NS	Guo et al. [30]
		2	NS	Huang et al. [32]
		3	sig	

Table 8.4a
IL-17 storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	35C	4 h	sig	Skogstrand et al. [36]
		RT	4 h	sig	
		4C	4 h	sig	
Serum	Heparin	−80C	36 months	sig	De Jager et al. [13]

Table 8.4b
IL-17 freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Plasma	Heparin	1	sig	De Jager et al. [13]
Serum	None	1	sig	Parkitny et al. [35]

Table 8.5a
TGFβ storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	EDTA	35C	48 h	NS	Skogstrand et al. [36]
		RT	48 h	sig	
		4C	48 h	NS	
Serum	None	37C	24 h	sig	Chaigneau et al. [26]

compared to endogenous cytokine studies where even 1 freeze/thaw cycle induced change [30,35].

Thavasu et al. [19] used recombinant TNFα, IFNα, IFNɣ, IL-1α, IL-1β, and IL-6. In most of these instances, their findings were consistent with others', however Table 4.1a and 4.1b emphasize that recombinant IL-6 appeared less stable than endogenous IL-6 when stored in plasma separated by EDTA or heparin. Table 5.2a also shows that recombinant IFNɣ is less stable than endogenous IFNɣ when refrigerated as serum or after separation with heparin or EDTA. Thavasu was the only researcher who looked at IFNα (Table 5.1), so it is difficult to draw conclusions about endogenous IFNα cytokine behavior.

Fraser et al' [20] study looked at recombinant IL-13 storage stability (Table 4.9a) and found when compared to endogenous IL-13 stored as serum, it did not go as long without significant concentration changes.

10. Conclusions

Although much of the existing research is conflicting, certain cytokines seem more stable when exposed to different storage temperatures or a different number of freeze/thaw cycles prior to quantification, such as IL-9, CXCL10, and eotaxin-1. Cytokines that are especially unstable or for which no clear consensus exists should be assayed as soon as possible after the blood sample is collected, such as IL-1RA, IL-4, and IL-

Table 8.5b
TGFβ freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/thaw cycles	Statistically significant?	Source
Serum	None	100	NS	Chaigneau et al. [26]

Table 8.6a
VEGF storage stability.

Sample type	Anticoagulant	Storage temp [C]	Time in storage	Statistically significant?	Source
Plasma	Heparin	4C	6 days	NS	Guo et al. [30]
Serum	None	4C	6 days	NS	Guo et al. [30]

Table 8.6b
VEGF freeze/thaw stability.

Sample type	Anticoagulant	# of freeze/ thaw cycles	Statistically significant?	Source
Plasma	Heparin	4	NS	Brøndum et al. [25]
		10	NS	Guo et al. [30]
Serum	None	10	NS	Guo et al. [30]
		2	NS	Huang et al.
		3	sig	[32]

5. The reasons some cytokines are more unstable than others may be related to the speed of degradation, the cytokine being secreted after blood has been collected, or the structure of the cytokine itself.

Some cytokines and chemokines have not been studied at all, and further work needs to be done to assure that storage mechanisms and freeze/thaw practices produce reliable results when quantifying cytokines. As there becomes more of a role for accurate cytokine measurement in research and for direct patient-care purposes, this body of research will help guide lab storage practices.

This paper, to our knowledge, is the first of its kind presenting a synthesized review of temperature stability of cytokines, organized by cytokine, which can be used for easy reference for the clinician or researcher to determine how blood samples need to be handled prior to cytokine quantification to assure the most accurate results.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] Bharat Aggarwal, Raj Puri, Human Cytokines: Their Role in Disease and Therapy, Blackwell Science Inc, Cambridge, MA, 1995.
[2] C.D. Fjell, S. Thair, J.L. Hsu, K.R. Walley, J.A. Russell, J. Boyd, Cytokines and signaling molecules predict clinical outcomes in sepsis, PLoS One 14 (2013) 8.
[3] A. Lin, G. Kenis, S. Bignotti, G.J.E. Tura, R. de Jong, et al., The inflammatory response system in treatment-resistant schizophrenia: increased serum interleukin-6, Schizophrenia Res. 32 (1998) 9–15.
[4] S. Bonaccorso, A. Lin, C. Song, R. Verkerk, G. Kenis, et al., Serotonin-immune interactions in elderly volunteers and in patients with Alzheimer's disease (DAT): lower plasma tryptophan availability to the brain in the elderly and increase serum interleukin-6 in DAT, Aging Clin. Exp. Res. 10 (1998) 316–323.
[5] S.A. De Serres, B.G. Mfarrej, M. Grafals, L.V. Riella, C.N. Magee, et al., Derivation and validation of a cytokine-based assay to screen for acute rejection in renal transplant recipients, Clin. J. Am. Soc. Nephrol. 7 (2012) 1018–1025.
[6] R. Saladino, M. Erikson, N. Levy, D. Bachman, G.R. Siber, G.R. Fleisher, Utility of interleukin-6 for diagnosis of invasive bacterial disease, Ann. Emerg. Med. 21 (1992) 1413–1417.
[7] M. Dy, A. Vazquez, J. Bertoglio, J. Théze, General aspects of cytokine properties and functions. The cytokine network and immune functions, Jacques Théze, Oxford University Press, Oxford, 1999.
[8] A. Wuyts, S. Struyf, P. Proost, J. Van Damme, "Chemokines." The cytokine network and immune functions, Jacques Théze, Oxford University Press, Oxford, 1999.
[9] N.T. Holland, M.T. Smith, B. Eskenazi, M. Bastaki, Biological sample collection and processing for molecular epidemiological studies, Mutat. Res. 543 (2003) 217–234.
[10] P. Tighe, O. Negm, I. Todd, L. Fairclough, Utility, reliability and reproducibility of immunoassay multiplex kits, Methods 61 (2013) 23–29.
[11] G.C. Keustermans, S.B.E. Hoeks, J.M. Meerding, B.J. Prakken, W. de Jager, Cytokine assays: an assessment of the preparation and treatment of blood and tissue samples, Methods 61 (2013) 10–17.
[12] H.J. Hansen, G. LaFontaine, E.S. Newman, M.K. Schwartz, A. Malkin, et al., Solving the problem of antibody interference in commercial sandwich immunoassays of carcinoembryonic antigen, Clin. Chem. 35 (1989) 146–151.
[13] W. de Jager, K. Bourcier, G.T. Rijkers, B.J. Prakken, V. Seyfert-Margolis, Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays, BMC Immunol. 10 (2009) 52.
[14] L.T. Hennø, E. Storjord, D. Christiansen, G. Bergseth, J.K. Ludviksen, et al., Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of the 27 multiplex assayed cytokines – consequences for defining reference values in healthy humans, Cytokine 97 (2017) 86–95.
[15] F.B. Vincent, H.T. Nim, J.P.W. Lee, E.F. Morand, J. Harris, Effect of storage duration on cytokine stability in human serum and plasma, Cytokine (2018) in press.
[16] B. Engelmann, S. Massberg, Thrombosis as an intravascular effector of innate immunity, Nat. Rev. Immunol. 13 (2013) 34–45.
[17] F. Gartner, S. Massberg, Blood coagulation in immunothrombosis – at the frontline of intravascular immunity, Sem. Immunol. 28 (2016) 561–569.
[18] C.A. Ray, R.R. Bowsher, W.C. Smith, V. Devanarayan, M.B. Willey, et al., Development, validation, and the implementation of a multiplex immunoassay for the simultaneous determination of five cytokines in human serum, J. Pharmaceut. Biomed. Anal. 36 (2005) 1037–1044.
[19] P.W. Thavasu, S. Longhurst, S.P. Joel, M.L. Slevin, F.R. Balkwill, Measuring cytokine levels in blood, J. Immunol. Methods 153 (1992) 115–124.
[20] S. Fraser, C. Fleener, K. Ogborne, C. Soderstrom, When close is not close enough: a comparison of endogenous and recombinant biomarker stability samples, Bioanalysis 7 (2015) 1355–1360.
[21] P. Riches, R. Gooding, B.C. Miller, A.W. Rowbottom, Influence of collection and separation of blood samples on plasma IL-1, IL-6 and TNF-α concentrations, J. Immunol. Methods 153 (1992) 125–131.
[22] A. Aguilar-Mahecha, M.A. Kuzyk, D. Domanski, C.H. Borchers, M. Basik, The effect of pre-analytical variability on the measurement of MRM-MS-Based mid- to high-abundance plasma protein biomarkers and a panel of cytokines, PLoS One 7 (2012) e38290.
[23] N. Aziz, P. Nishanian, R. Mitsuyasu, R. Detels, J.L. Fahey, Variables that affect assays for plasma cytokines and soluble activation markers, Clin. Diag. Lab. Immunol. 6 (1999) 89–95.
[24] N. Aziz, R. Detels, J.J. Quint, Q. Li, D. Gjertson, A.W. Butch, Stability of cytokines, chemokines and soluble activation markers in unprocessed blood stored under different conditions, Cytokine 84 (2016) 17–24.
[25] L. Brøndum, B.S. Sørensen, J.G. Eriksen, S.L. Mortensen, S. Lønbro, et al., An evaluation of multiplex bead-based analysis of cytokines and soluble proteins in archived lithium heparin plasma, EDTA plasma, and serum samples, Scand. J. Clin. Lab. Invest. 76 (2016) 601–611.
[26] C. Chaigneau, T. Cabioch, K. Beaumont, F. Betsou, Serum biobank certification and the establishment of quality controls for biological fluids: examples of serum biomarker stability after temperature variation, Clin. Chem. Lab. Med. 45 (2007) 1390–1395.
[27] L. Flower, R.H. Ahuju, S. Humphries, V. Mohamed-Ali, Effects of sample handling on the stability of interleukin 6, tumour necrosis factor-α and leptin, Cytokine 12 (2000) 1712–1716.
[28] A. Friebe, H.D. Volk, Stability of tumor necrosis factor α, interleukin 6, and interleukin 8 in blood samples of patients with systemic immune activation, Arch. Pathol. Lab. Med. 132 (2008) 1802–1806.
[29] C. Graham, R. Chooniedass, W.P. Stefura, L. Lotoski, P. Lopez, et al., Stability of pro- and anti-inflammatory immune biomarkers for human cohort studies, J. Transl. Med. 15 (2017) 53.
[30] G.H. Guo, J. Dong, X.H. Yuan, Z.N. Dong, Y.P. Tian, Clinical evaluation of the levels of 12 cytokines in serum/plasma under various storage conditions using evidence biochip arrays, Mol. Med. Rep. 7 (2013) 775–780.
[31] F.S. Hosnijieh, E.J.M. Krop, L. Portengen, C.S. Rabkin, J. Linselsen, et al., Stability and reproducibility of simultaneously detected plasma and serum cytokine levels in asymptomatic subjects, Biomarkers 15 (2010) 140–148.
[32] W.Y. Huang, T.J. Kemp, R.M. Pfeiffer, L.A. Pinto, A. Hildesheim, M.P. Purdue, Impact of freeze-thaw cycles on circulating inflammation marker measurements, Cytokine 95 (2017) 113–117.
[33] G. Kenis, C. Teunissen, R. de Jongh, E. Bosmans, H. Steinbusch, M. Maes, Stability of interleukin 6, soluble interleukin 6 receptor, interleukin 10 and CC16 in human serum, Cytokine 19 (2002) 228–235.
[34] O.M.P. Palmer, M. Carter, C.C.H. Chang, N. Lucko, V.M. Jackson, et al., Effects of transport temperature on the stability of inflammatory, hemostasis, endothelial function, and oxidative stress plasma biomarker concentrations, Shock 47 (2017) 715–719.
[35] L. Parkitny, J.H. McAuley, P.J. Kelly, F. Di Pietro, B. Cameron, G.L. Moseley, Mulitplex cytokine concentration measurement: How much do the medium and handling matter? Mediators of Inflamm (2013).
[36] K. Skogstrand, C.K. Ekelund, P. Thorsen, I. Vogel, B. Jacobsson, et al., Effects of blood sample handling procedures on measurable inflammatory markers in plasma, serum and dried blood spot samples, J. Immunol. Methods 336 (2008) 78–84.
[37] R.P. van Waateringe, A.C. Muller Kobold, J.V. van Vliet-Ostaptchouk, M.M. van der

Klaw, J. Koerts, G. Anton, et al., Influence of storage and inter- and intra-assay variability on the measurement of inflammatory biomarkers in population-based biobanking, Biopres. Biobank 15 (6) (2017) 512–518.
[38] J. Zander, M. Bruegel, A. Kleinhampel, S. Becker, S. Petros, et al., Effect of biobanking conditions on short-term stability of biomarkers in human serum and plasma, Clin. Chem. Lab. Med. 52 (2014) 629–639.
[39] A. Mantovani, The interleukin-1 system. The cytokine network and immune functions, Jacques Théze, Oxford University Press, Oxford, 1999.
[40] H. Okamura, M.T. Lotze, H. Tsutsui, S. Kashiwamura, H. Ueda, T. Yoshimoto, K. Nakanishi, “Interleukin-18.” The Cytokine Handbook. fourth ed. Michael T Lotze, Academic Press, Chicago, 2003.
[41] J. Vilcek, The cytokines: An overview, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[42] T. Enzler, G. Dranoff, Granulocyte-macrophage colony-stimulating factor, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[43] C.C. Kok, G.T. Schwenger, R.I.W. Osmond, D.L. Urwin, C.J. Sanderson, “Interlekin-5.”. The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[44] J.X. Lin, W.J., Leonard, “Interleukin-2.”, The Cytokine Handbook, 4th ed., Michael T Lotze, Academic Press, Chicago, 2003.
[45] H. Okada, J. Banchereau, M.T. Lotz, “Interleukin-4.” The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[46] P.K.E. Trinder, M.J. Maeurer, “Interleukin-7.”, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[47] J.C. Renauld, J. Van Snick, Interleuin-9, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[48] M.R. Shurin, I.L. Tourkova, H. Hackstein, G.V. Shurin, Interleukin-15 and 21.”, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[49] G. Grünig, J.E. de Vries, R. de Waal Malefyt, “Interleukin-13.”, The Cytokine Handbook, fourth ed., Michael T Lotze. Academic Press, Chicago, 2003.
[50] P. Kalinski, W.J. Storkus, A.W. Thomson, M.T. Lotze, Interleukin-12 Family. The Cytokine Handbook. fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[51] S.M. White, D.J. Tweardy, “Granulocyte colony-stimulating factor.”, The Cytokine Handbook, fourth ed., Michael T Lotze. Academic Press, Chicago, 2003.
[52] J.M. Soos, B.E. Szente, “Type I interferons.” The Cytokine Handbook, fourth ed., Michael T Lotze. Academic Press, Chicago, 2003.
[53] G.H. Schreiber, R.D. Schreiber, “Interferon-ɣ”. The Cytokine Handbook, 4th ed., Michael T Lotze. Academic Press, Chicago, 2003.
[54] Y.Z. Ding, S. Fu, D. Zamarin, J. Bromberg, “Interleukin-10.” The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[55] H. Wang, C.J. Czura, K.J. Tracey. “Tumor necrosis factor.”, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[56] S.W. Granger, C.F. Ware, “Lymphotoxins.”, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[57] N. Mukaida, S.A. Ketlinsky, K. Matsushima, “Interleukin-8 and other CXC chemokines.”, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[58] K.C. Wilson, D.M. Center, W.W. Cruikshank, “Interleukin-16.”, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[59] T. Kishimoto, Interleukin-6 family, The Cytokine Handbook, 4th ed., Michael T Lotze. Academic Press, Chicago, 2003.
[60] D.C. Lee, C.L. Hinkle, L.F. Jackson, S. Li, S. Wohler Sunnarborg, “EGF family ligands.”, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.
[61] M.A. Antonysamy, M. Numasaki, Interleukin-17, The Cytokine Handbook, fourth ed., Michael T Lotze. Academic Press, Chicago, 2003.
[62] R.J. Leichlader, A.B. Roberts, “Transforming growth factor-β.”, The cytokine network and immune functions. Jacques Théze. Oxford University Press, Oxford, 1999.
[63] D.I. Gabrilovich, M.M. Dikov, “Vascular endothelial growth factor.”, The Cytokine Handbook, fourth ed., Michael T Lotze, Academic Press, Chicago, 2003.